QUANTITATIVE DETERMINATION OF SOME CONSTITUENT LIPIDS IN HUMAN DENTIN

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FOREWORD

This report was prepared by the following personnel at the USAF School of Aerospace Medicine:

(10) by THOMAS R. DIRKSEN, and ~~Captain, USAF, DC*~~
KENNETH G. IKELS, ~~Mst.~~†

(*Experimental Dentistry Department; †Physiology Branch)

The authors are grateful for the technical assistance of Clarence F. Theis, A2C John E. Taylor, A3C William B. Jacobs, and A3C Clemmon L. Woodard.

ABSTRACT

A method is described for the extraction of dentin lipids and the separation of these lipids into six components (cholesterol esters, free cholesterol, triglycerides, diglycerides, monoglycerides, and phospholipids).

Essentially, the method requires the decalcification of dentin using EDTA and subsequent extraction with diethyl ether, ethanol, and chloroform. The lipid extract is separated into six components by a small silicic acid column and the effluents are quantitated colorimetrically.

This technical documentary report has been reviewed and is approved.

Robert B Payne

ROBERT B. PAYNE
Colonel, USAF, MSC
Chief, Operations Division

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1. INTRODUCTION

By the paper chromatographic technics developed by Marinetti and Stotz (1, 2), cholesterol esters, triglycerides, nonesterified fatty acids, cholesterol, diglycerides, monoglycerides, and various phospholipids were earlier identified as constituents of human dentin (3, 4). This paper describes methods whereby the above-mentioned dentin lipids, with exception of the nonesterified fatty acids, may be quantitated by separation on microsilicic acid columns and subsequent colorimetric determinations (5).

2. METHODS AND MATERIALS

Preparation of dentin

Human teeth, immediately after extraction, were washed in distilled water and frozen with Dry Ice. As dentin was required, the teeth were carefully cleaned and, if present, carious portions of the teeth were removed so that only sound dentin was utilized. The procedures of Leopold et al. (6) were incorporated to isolate dentin. After reducing the sample material with a steel, plunger-type mortar and pestle to pass a 200-mesh sieve, metal filings were removed magnetically and the material was dried to constant weight in a nitrogen-filled desiccator. Lipid materials were initially extracted from the sound dentin, while subsequent studies employed dentin decalcified by EDTA (ethylenediamine tetra-acetic acid).

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Extraction of dentin lipids

Sound dentin. Reagent-grade chloroform was redistilled prior to use. Ten-gram samples of 200-mesh dentin and 30 ml. chloroform : methanol (2 : 1) were placed into glass-stoppered tubes and shaken on a wrist-action shaker for one hour. The mixtures were centrifuged and the solvents decanted; 30 ml. chloroform were added to the solutes and the mixtures were again shaken for 1 hour. After centrifugation and decantation, another quantity of 30 ml. chloroform was added and a final shaking was employed for 20 hours. The solvents for each 10-gm. sample, along with several 5-ml. chloroform washes of the glassware and sample material, were combined and filtered through a medium-sintered glass funnel into glass-stoppered graduated cylinders.

To remove nonlipid impurities, the solvents were shaken with 20 ml. of distilled water for one minute and allowed to stand overnight. As much as possible of the upper aqueous phase was removed by suction, 2 ml. methanol were added, and the solvent layer was transferred to a round-bottom flask for evaporation under vacuum. The lipid material was dissolved in chloroform and transferred to tared flasks; the total weight was determined after equilibrium had been obtained in a nitrogen-filled desiccator. The samples were then dissolved in petroleum ether and applied to the silicic acid columns.

EDTA-decalcified dentin. Dentin was decalcified by placing 5-gm. samples into dialysis bags previously extracted for 30 minutes with distilled water, followed by one hour extraction with chloroform : methanol (2 : 1). To the samples, 10 ml. of 15% EDTA (pH 7.4) were added and the whole system immersed in 1,500

ml. of the same fluid with constant stirring at 4° C. for 10 days. The decalcified matrix was dialyzed against distilled water for two days, transferred to glass-stoppered tubes, centrifuged, and the supernatant transferred to glass-stoppered graduated cylinders.

As concurrent studies utilizing paper chromatography (4) indicated, not all dentin lipids are extracted with chloroform : methanol (in particular some phospholipids); the procedures for extracting EDTA-decalcified dentin were, therefore, altered for lipid determination. These alterations employed reagent-grade diethyl ether, which was redistilled in glass prior to use, and absolute ethyl alcohol. The decalcified matrix representing 5 gm. original material was extracted 30 minutes with each of the following: (a) 30 ml. diethyl ether : ethanol (2 : 1), (b) 30 ml. diethyl ether : ethanol (2 : 1), and (c) 30 ml. diethyl ether. The solvents were transferred to a graduated cylinder containing the water from within the dialysis bag and shaken vigorously. A biphasic system was obtained after several hours, whereupon the ether layer was transferred to a round-bottom flask for evaporation.

During this time, 30 ml. chloroform was added to the matrix, and the resulting mixture was shaken for one hour. After centrifugation, the solvent was decanted into the same graduated cylinder and the washing procedure was repeated. The chloroform layer was added to the ether-soluble material in the round-bottom flask and evaporated. Following evaporation, the lipid material was dissolved in chloroform and transferred to tared flasks and the total weight determined. When the altered extraction procedures were applied to sound dentin, only slight differences in results were noted as compared with chloroform : methanol.

Column chromatography of dentin lipids

The method of chromatographic separation of dentin lipids is essentially that of Ikels (5). Minor alterations were made in the amounts of eluents and the concentration of diethyl ether. After the column had been charged with the dentin lipids, 25 ml. of 1%, 30 ml. of 4%, 45 ml. of 8%, and 25 ml. of 25% diethyl ether in petroleum ether and 15 ml. 100% diethyl ether were passed through the column and collected in separate fractions. The above-named solutions eluted cholesterol esters, triglycerides, free cholesterol, diglycerides, and monoglycerides, respectively. Phospholipids were eluted with 15 ml. each of 25% and 50% methanol : chloroform and 15 ml. absolute methanol. Elution patterns were determined both by paper chromatography (1, 2) and by colorimetric determinations of eluents collected in 5-ml. fractions.

3. RESULTS

The total lipid extracts and the different lipid classes separated by column chromatography for seven 10-gm. samples of dentin not treated with EDTA are recorded in table I. Although considerable variation was noted from sample to sample, average values were calculated as a means of comparison with EDTA-decalcified samples and previous studies (6, 7).

TABLE I

Non-EDTA-treated dentin lipid components

Sample	Total weight	Cholesterol esters	Cholesterol free	Triglycerides	Diglycerides	Monoglycerides	Phospholipids
	(milligrams per 100 gm.)						
1	46.2	2.54	3.24	1.05	0.61		2.10
2	48.6	2.44	3.27	1.19	0.69		1.58
3	43.2	2.73	2.72	2.58	0.81		1.43
4	45.1	3.46		2.39	0.83		2.05
5	30.0	2.43	2.36	1.27	0.51		
6	32.2	3.32	4.43	1.30	0.93	0.45	2.73
7		3.32	4.49	1.38	0.84	0.45	2.35
Mean	40.8	2.89	3.42	1.59	0.75	0.45	2.04

TABLE II

EDTA-decalcified dentin components

Sample	Total weight	Cholesterol esters	Cholesterol free	Triglycerides	Diglycerides	Monoglycerides	Phospholipids
	(milligrams per 100 gm.)						
1	169.6	3.68	5.76	1.53	1.10	0.48	5.08
2	186.4	3.11	6.35	1.37	0.63	0.38	6.05
3	224.0	3.41	6.94	1.20	1.12	0.98	4.45
4	162.2	4.11	6.20	1.30	1.31	1.38	5.60
5	153.2	5.08	6.79	2.13	1.25	0.81	3.94
6	164.4	4.77	7.13	2.11	1.51	0.74	4.45
Mean	176.6	4.03	6.53	1.61	1.15	0.79	4.94

Fully calcified human dentin yielded an average concentration of 40.88 mg. per 100 gm. dentin of chloroform : methanol soluble material. The concentration of individual components in milligrams per 100 gm. dentin was as follows: cholesterol in esterified form, 1.71; triglycerides, 1.59; cholesterol, 3.42; diglycerides, 0.74; monoglycerides, 0.45; and phospholipid phosphorus, 0.082. Application of conversion factors commonly used for blood serum (cholesterol esters, 1.69; phospholipids, 25), plus the other colorimetrically determined constituents, gave a total weight of 11.13 mg. of lipids per 100 gm. dentin. This amounted to approximately 27 percent of the column charge.

Better yields of lipids were obtained when EDTA-decalcified dentin was extracted (see table II). Ether : ethanol : chloroform extraction of the decalcified matrix of six 5-gm. samples of dentin yielded an average of 176.6 mg. per 100 gm. dentin. The separation of the lipid material on silicic acid columns indicating the concentration of individual components in milligrams per 100 gm. dentin was as follows: cholesterol in esterified form, 2.50; triglycerides, 1.60; cholesterol, 6.50; diglycerides, 1.15; monoglycerides, 0.80; and phospholipid phosphorus, 0.88. Conversion factors used for blood serum in calculating total weights of cholesterol esters and phospholipids gave a value of 18.98 mg. of lipid material per 100 gm. dentin or 10.7 percent of the column charge.

4. DISCUSSION

Leopold et al. (6) determined 0.024% total cholesterol in dentin by an alkaline hydrolysis : petroleum ether extraction. Hess et al. (7), extracting sound dentin with alcohol : ether, found 0.36 percent total lipids in excess of cholesterol of which 0.014 percent were phospholipids. In comparing past findings with present results, we found considerably lower yields in our determinations. We have also noted a wide range of values from sample to sample, particularly for the nondecalcified dentin. This variation in results may be due to any or all of the following reasons:

a. Dentin was ground to 200-mesh size with no effort to standardize the lower limit of particle size. Nondecalcified samples presenting a greater surface area to solvent action could conceivably give higher lipid yields.

b. Various amounts and types of contaminants are soluble in the commonly used lipid solvent (8), and phospholipids seemingly have the ability to solubilize many lipid impurities (9). In this respect, gravimetric determinations of total lipid content without some means of purification may be subject to error.

c. Lipid substances are subject to degradation, and lengthy grinding and decalcification procedures would not be without effect.

d. The age, type, and condition of teeth in any particular sample may have an effect on the content and type of lipid material present (10).

e. The inclusion of even a small amount of pulpal tissue in the sample material would alter the results (11).

The major lipid components of dentin recovered during column chromatography amounted to 10 to 30% of the total column charge. We speculate that the amounts of material not recovered from the column reflect the presence of impurities and of lipids other than those quantitated. One such group of lipids might be the free fatty acids, which are known constituents of dentin and which are eluted from the silicic acid column with the triglyceride and free cholesterol fractions. The presence of other unidentified substances was indicated by paper chromatography, but neither the amounts nor the identity of these is known.

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